

# Relationship between soil densities of *Aspergillus* species and colonization of wounded peanut seeds

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**Abstract:** Soil is a reservoir for *Aspergillus flavus* and *A. parasiticus*, fungi that commonly colonize peanut seeds and produce carcinogenic aflatoxins. Densities of these fungi in soil vary greatly among fields and may influence the severity of peanut infection. This study examined the relationship between soil density of *Aspergillus* species and the incidence of peanut seed colonization under laboratory conditions. Viable peanut seeds were wounded and inoculated with 20 soils differing in composition and density of *Aspergillus* species and were then incubated for 14 days at 37 °C (seed water activity = 0.92). The effect of soil density of individual section *Flavi* species (*A. flavus* strains L and S, *A. parasiticus*, *A. caelatus*, and *A. tamarii*), section *Nigri*, and *A. terreus* on the incidence of seed colonization was best expressed as a function of exponential rise to maximum. Exponential curves often rose to maximum percentages of seed colonization by section *Flavi* species that were well below 100% despite high species densities in some soils. Competition primarily among section *Flavi* species may explain the reduced incidences of seed colonization. An average of two or fewer propagules of each *Aspergillus* species in the soil at the wound site was required for colonization of 20% of peanut seeds. Other fungal species were capable of invading peanut seeds only when soil densities of sections *Flavi* and *Nigri* species were low.

**Key words:** aflatoxin, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, fungal competition.

**Résumé :** Le sol est un réservoir de *Aspergillus flavus* et *A. parasiticus*, des champignons qui colonisent couramment les graines d'arachide et produisent des aflatoxines cancérogènes. Les densités de ces champignons dans le sol varient grandement parmi les champs et pourraient influencer la gravité de l'infection des arachides. Cette étude a examiné la relation entre la densité d'espèces d'*Aspergillus* dans le sol et l'incidence de la colonisation de graines d'arachide dans des conditions de laboratoire. Les graines d'arachide viables ont été endommagées et inoculées avec 20 sols de compositions et de densités d'*Aspergillus* différentes, puis incubées pendant 14 jours à 37 °C (activité de l'eau des graines = 0,92). L'impact de la densité d'espèces individuelles de la section *Flavi* (souches L et S de *A. flavus*, *A. parasiticus*, *A. caelatus* et *A. tamarii*), de la section *Nigri* et *A. terreus* sur l'incidence de la colonisation des graines fut exprimée de la meilleure façon sous forme d'une fonction exponentielle à montée au maximum. Les courbes exponentielles ont souvent monté jusqu'à leurs pourcentages maximums de colonisation des graines par des espèces de la section *Flavi* qui étaient bien en deçà de 100 % malgré la densité élevée des espèces dans certains sols. Une compétition principalement parmi les espèces de la section *Flavi* pourrait expliquer les incidences réduites de colonisation des graines. Une moyenne de deux propagules ou plus d'espèces d'*Aspergillus* du sol au site de la lésion fut nécessaire à la colonisation de 20 % des graines d'arachide. D'autres espèces de champignons furent capables d'envahir la graine d'arachide seulement lorsque les densités dans le sol des espèces des sections *Flavi* et *Nigri* étaient basses.

**Mots clés :** aflatoxine, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, compétition de champignons.

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## Introduction

*Aspergillus flavus* and *A. parasiticus*, fungi belonging to *Aspergillus* section *Flavi*, commonly invade oil-rich seeds and grain, such as peanuts, corn, cottonseed, and tree nuts, in which they produce the carcinogenic aflatoxins (Diener et al. 1987; Payne 1998). Aflatoxins show considerable toxicity in some animals (Hussein and Brasel 2001), and epidemio-

logical studies of human populations suggest that prolonged ingestion of aflatoxins results in an increase in hepatocellular carcinoma when interacting with hepatitis viruses (Turner et al. 2002). The cost of managing aflatoxins in agricultural produce is enormous (Robens and Cardwell 2005). In the southeastern United States alone, the peanut industry spends an average of \$26 million annually for aflatoxin management (Lamb and Sternitzke 2001).

Another mycotoxin, cyclopiazonic acid, is also produced by *A. flavus* (Horn and Dörner 1999) and often co-occurs with aflatoxins in agricultural commodities (Urano et al. 1992). *Aspergillus flavus* has been subdivided into two morphotypes: the L strain, which produces large sclerotia >400 µm in diameter, and the S strain, described as variety *parvisclerotigenus* (Saito and Tsuruta 1993), which produces numerous small sclerotia <400 µm in diameter (Cotty 1989).

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Other section *Flavi* species in crops include *A. tamarii*, *A. caelatus* (Horn 1997), and *A. alliaceus* (teleomorph = *Petromyces alliaceus*). *Aspergillus tamarii* and *A. alliaceus* produce cyclopiazonic acid and ochratoxin A, respectively (Bayman et al. 2002; Dörner 1983).

Soil is a reservoir for *A. flavus*, *A. parasiticus*, and other species from section *Flavi* (Horn and Dörner 1998; Horn et al. 1995). In contrast to aerial crops, peanuts (*Arachis hypogaea*) with their subterranean growth habit are exposed directly to soil populations of these fungi (Horn 2005a). Peanut seeds are most susceptible to invasion by *A. flavus* and *A. parasiticus* under conditions of plant drought stress and elevated soil temperatures (Blankenship et al. 1984; Sanders et al. 1981). Reduced water activity ( $A_w$ ) of peanut seeds during drought suppresses phytoalexin production and thereby increases susceptibility of seeds to invasion by aflatoxigenic fungi (Dörner et al. 1989). Pod and seed damage also greatly increase invasion by *A. flavus* and *A. parasiticus* and subsequent aflatoxin formation (Dowell et al. 1990; Sanders et al. 1985). In the United States, considerable damage is caused by the lesser cornstalk borer (*Elasmopalpus lignosellus*) (Lynch 1984; Lynch and Wilson 1991). Other arthropods, such as white grubs (scarab beetle larvae), termites, and millipedes, are responsible for damage to peanut pods and seeds in tropical regions (Lynch and Mack 1995).

One of the perplexing problems in aflatoxin research concerns the influence of soil density of aflatoxigenic fungi on crop infection, which ultimately may influence the severity of aflatoxin contamination (Horn 2005a). The direct contact of peanuts with soil populations makes this an ideal crop for examining the relationship between population density and seed colonization. Based on field observations, Griffin and Garren (1974) suggested that *A. flavus* is capable of colonizing peanut fruits at very low soil densities. However, more controlled conditions for critical variables such as pod and seed damage, soil temperature, and seed water activity are necessary to accurately assess the effect of soil population density on seed colonization.

Horn (2005b) developed a laboratory procedure in which viable fungus-free peanut seeds are wounded and inoculated with soil directly from the field under specific conditions of temperature and seed water activity. Species from section *Flavi* were shown to preferentially colonize wounded peanut seeds despite low soil densities relative to the total filamentous fungal population. However, only two soils were examined, and no attempt was made to determine the effect of propagule density in soil on seed colonization. In the present study, 20 soils differing in composition and density of *Aspergillus* species were used for inoculating wounded peanut seeds to characterize the effect of fungal density on the colonization of seeds.

## Materials and methods

### Soil populations

Soil was collected on 18 April and 5 June 2002 and on 31 March 2003 from 20 different locations in southwestern Georgia, USA (Table 1). Forested locations were covered with hardwood and (or) pine trees; fallow fields consisted of herbaceous plants with widely scattered pines (1–3 m tall);

and cultivated fields were planted with peanuts, corn, cotton, or wheat during the previous growing season. Approximately 1 kg of soil was collected from the top 5 cm, thoroughly mixed, and stored in a sealed plastic bag at 5 °C. Soils were used within 3 weeks for quantifying fungal populations and for inoculating seeds.

Population densities of *Aspergillus* species were determined by dilution plating three subsamples (3.3 g each) of soil onto modified dichloran – rose bengal medium (5 plates/subsample) and incubating for 3–4 days at 37 °C, as described by Horn and Dörner (1998). Unmodified dichloran – rose bengal plates were used for enumerating total filamentous fungi (5 days, 25 °C) from subsamples (Horn et al. 1994); those same plates were incubated an additional 2–3 days (30 °C) for quantifying *Eupenicillium ochrosalmoneum* (Horn 2005b). *Aspergillus* species were identified either directly from dilution plates or by subculturing to slants of Czapek agar (Horn and Dörner 1998). Biseriate species belonging to *Aspergillus* section *Nigri* were not differentiated and were recorded as section *Nigri*. All fungal densities are based on the wet mass of the final soil preparation used for inoculating seeds.

### Peanut seed inoculations

Peanut plants ('Georgia Green') were irrigated and grown using standard cultivation practices (Guillebeau 2004). Plants were dug with a digger–inverter on 13 September 2001 (5.4 km southwest of Dawson, Terrell County, Georgia) and 8 September 2002 (2.1 km north of Sasser, Terrell County). Pods were immediately handpicked and abraded with a wet impact blaster to clean and determine pod maturity (Williams and Drexler 1981; Williams and Monroe 1986). Mature pods (black and brown maturity classes) were dried for 14 days at ambient temperature on a forced-air drier and then stored at 5 °C and used within 1 year. The viability of 100 seeds from each of the two peanut harvests was examined after 1 year of storage, using the tetrazolium method (Peters 2000). Cotyledon and embryo tissue from all 200 seeds were shown to be viable (Horn 2005b).

Undamaged peanut pods were surface sterilized for 2 min in 2.0% sodium hypochlorite, followed by three sterile water rinses. Seeds were aseptically removed from the pods and were rehydrated, wounded, and inoculated with soil as described by Horn (2005b). Briefly, seeds were placed in four-section plates in which two compartments contained two seeds each and the other two compartments contained NaCl solution ( $A_w = 0.93$ ). Plates were incubated for 7 days at 37 °C over NaCl solution in covered desiccator jars, resulting in seed water activities of  $0.92 \pm 0.002$  ( $\pm$ SD;  $n = 10$ ), as measured with a Series 3TE water activity meter (Decagon Devices, Pullman, Wash.) at 37 °C. Cotyledons of seeds without visible fungal colonization were then aseptically wounded with a cork borer and dissecting needle (3 mm diameter wound to 1 mm depth), and wounded seeds were glued to the bottom of the plates. Soil inoculum consisted of a near-saturated paste (33 g soil plus 2.0–9.0 mL distilled water, depending upon soil texture and initial moisture content); wounds were inoculated with  $6.8 \pm 1.55$  mg of soil paste ( $\pm$ SD;  $n = 60$ ) with a small spatula. Forty seeds were inoculated with each soil and were incubated an additional

**Table 1.** Fungal densities and proportions in Georgia soils used for inoculating wounded peanut seeds.

Field No.	County	Land use	<i>Aspergillus</i> section <i>Flavi</i> (CFU/g)	% <i>Aspergillus</i> section <i>Flavi</i> <sup>a</sup>						Total filamentous fungi (10 <sup>4</sup> CFU/g)	Relative density of section <i>Flavi</i> (%) <sup>b</sup>
				F-L	F-S	P	C	T	A		
1	Upson	Forested	2±3.2			100				20±1.0	<0.001
2	Randolph	Forested	2±0.0	100						47±4.4	<0.001
3	Terrell	Forested	3±1.1	60		40				68±5.1	<0.001
4	Terrell	Fallow	6±6.0			100				10±1.1	<0.01
5	Sumter	Forested	19±10.6	50		50				86±12.6	<0.01
6	Macon	Fallow	61±8.1	31		69				18±1.6	0.03±0.002
7	Stewart	Cultivated	111±4.6	29	1	60	10			4±1.7	0.29±0.101
8	Stewart	Cultivated	175±49.3	7	13	79	1			4±0.5	0.47±0.064
9	Calhoun	Cultivated	226±30.9	32		12	51	5		23±4.2	0.10±0.034
10	Terrell	Cultivated	251±164.6	12	23	39	15	11		21±0.3	0.12±0.076
11	Terrell	Cultivated	269±110.0	6		80	12	2		8±0.8	0.33±0.145
12	Terrell	Cultivated	316±125.7	23	6	22	43	6		21±3.3	0.16±0.081
13	Sumter	Cultivated	429±24.0	69		24		7		10±2.0	0.43±0.101
14	Crawford	Cultivated	400±35.5		99	1				12±1.4	0.33±0.008
15	Randolph	Cultivated	491±57.7	37		49	11	3		8±1.2	0.62±0.160
16	Webster	Cultivated	629±74.9	61		39				7±0.2	0.92±0.092
17	Terrell	Cultivated	1128±222.7	28	2	30	10	3	27	13±1.2	0.84±0.210
18	Taylor	Cultivated	1225±192.7	2	88	6		4		13±0.9	0.96±0.108
19	Terrell	Cultivated	1335±258.3	32	14	16	15	4	19	11±0.7	1.19±0.248
20	Terrell	Cultivated	1733±263.9	24	21	43	8	4		22±6.8	0.81±0.220

**Note:** Values are the mean±SD ( $n=3$  subsamples) and are based on wet mass of final soil preparation used for inoculating seeds.

<sup>a</sup>Percentages of section *Flavi* are based on means of three subsamples. F-L, *A. flavus* L strain; F-S, *A. flavus* S strain; P, *A. parasiticus*; C, *A. caelatus*; T, *A. tamarii*; A, *A. alliaceus*.

<sup>b</sup>Percentages of total filamentous fungi.

14 days at 37 °C. Twenty-four uninoculated wounded seeds served as controls in each of the three experiments consisting of six or seven soils. None of the control seeds ( $n = 72$ ) showed fungal colonization by the end of the experiments.

Peanut seeds were examined with a stereomicroscope for fungal colonization at 4, 7, and 14 days following wound inoculation (Horn 2005b). With the exception of *A. alliaceus*, species from section *Flavi* could not be distinguished when sporulating on peanut seeds and were subcultured to Czapek agar slants for identification. From one to ten transfers were made from each seed, depending upon the extent of colonization. Section *Nigri* and *A. terreus* were identified directly on the seed; other *Aspergillus* species and fungal genera were identified as described by Horn (2005b).

## Statistics

Linear regressions and best-fit nonlinear regressions examining the relationships between soil densities of *Aspergillus* species and incidences of seed colonization were analyzed with SigmaPlot, Version 7.0 (Jandel Scientific, San Rafael, Calif.). Quadratic regressions examining the interactions between soil densities of section *Flavi* species and their competitors (other section *Flavi* species and section *Nigri*) in relation to the incidence of seed colonization were analyzed with the SAS statistical package, Version 8.2 (SAS Institute Inc., Cary, N.C.).

## Results

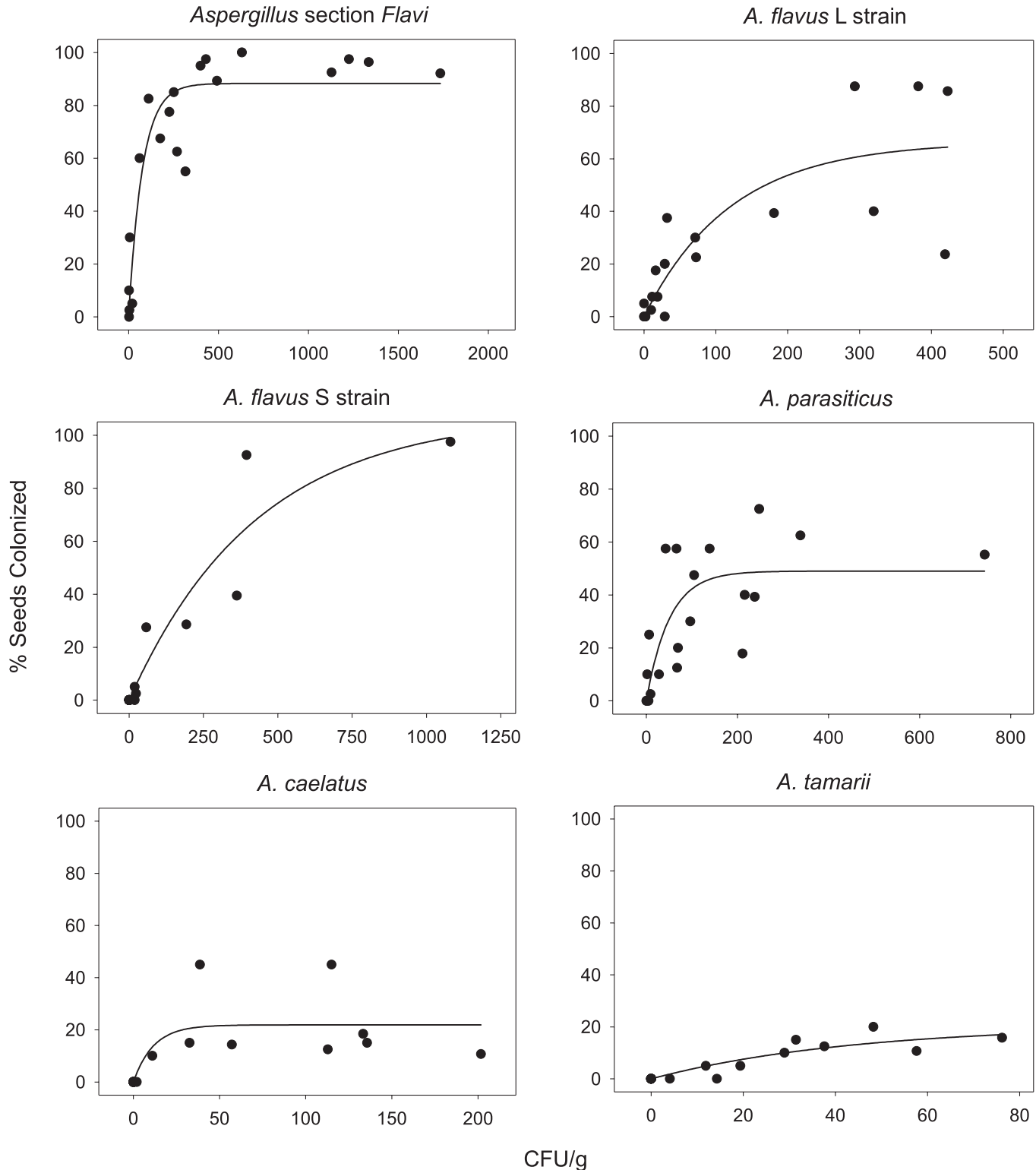
### Soil populations

*Aspergillus* section *Flavi* in soils used for inoculating the wounds of peanut seeds differed considerably in species composition, density, and relative density (percentage of total filamentous fungi) (Table 1). Soils from forested locations and fallow fields showed lower absolute and relative densities of section *Flavi* than soils from cultivated fields. All fields showed relative densities of <1.0% for section *Flavi*, with the exception of Field 19 in which the relative density was 1.19%.

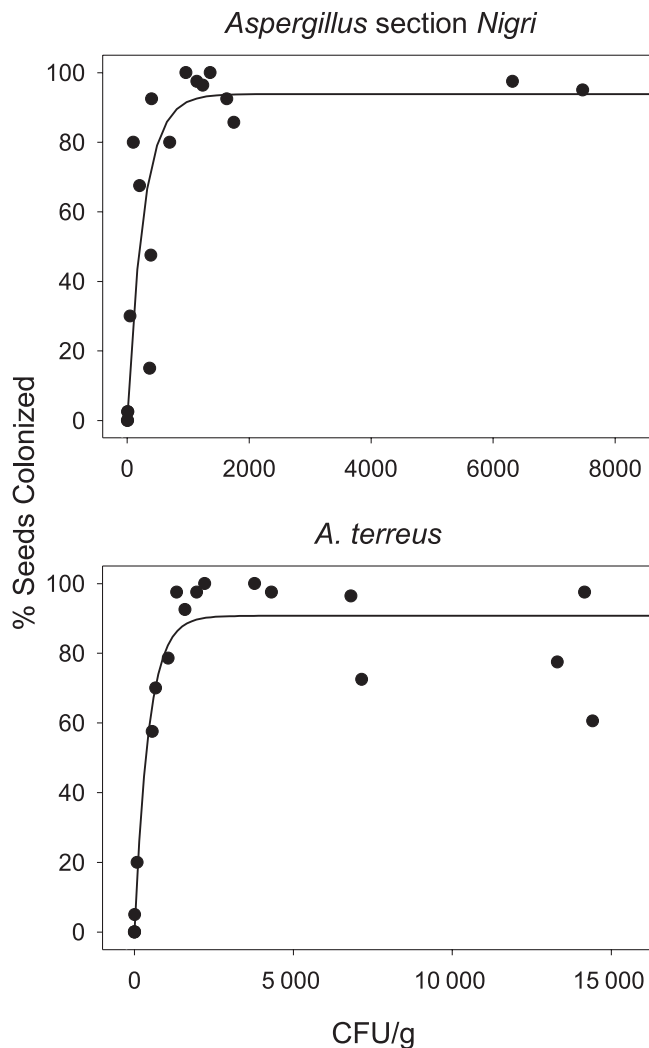
### Effect of soil fungal density on seed colonization

The incidence of peanut seed colonization by *Aspergillus* section *Flavi* (combined species) when regressed on propagule density in 20 different soils was highly significant ( $r^2 = 0.86$ ;  $P < 0.0001$ ), and the relationship was best expressed as a function of exponential rise to maximum (Fig. 1). Individual species within section *Flavi*, including *A. flavus* L and S strains, *A. parasiticus*, *A. caelatus*, and *A. tamarii*, also were characterized by exponential regressions (Fig. 1). *Aspergillus alliaceus* (not shown) was detected in only two soils (249 and 301 CFU/g) and was observed on seeds (2.5%–37.5%) inoculated with four soils. These limited data were best described by the linear function  $y = 0.16 + 0.0907x$  ( $r^2 = 0.81$ ;  $P < 0.0001$ ). Soils from forested locations and fallow

**Fig. 1.** Best-fit regression curves (exponential rise to maximum) for *Aspergillus* section *Flavi* (all species combined) and for individual species within the section showing the effect of fungal density in 20 soils on the incidence of peanut seed colonization. Soil densities are based on the wet mass of the final soil preparation used for inoculating seeds; percentages of seeds colonized are based on 40 seeds per soil. *Aspergillus* section *Flavi*:  $y = 88.32(1 - e^{-0.0131x})$ ,  $r^2 = 0.86$  ( $P < 0.0001$ ); *A. flavus* L strain:  $y = 66.46(1 - e^{-0.0082x})$ ,  $r^2 = 0.71$  ( $P < 0.0001$ ); *A. flavus* S strain:  $y = 107.74(1 - e^{-0.0023x})$ ,  $r^2 = 0.91$  ( $P < 0.0001$ ); *A. parasiticus*:  $y = 49.06(1 - e^{-0.0198x})$ ,  $r^2 = 0.55$  ( $P = 0.0002$ ); *A. caelatus*:  $y = 21.88(1 - e^{-0.0859x})$ ,  $r^2 = 0.60$  ( $P < 0.0001$ ); *A. tamarii*:  $y = 20.67(1 - e^{-0.0227x})$ ,  $r^2 = 0.86$  ( $P < 0.0001$ ).

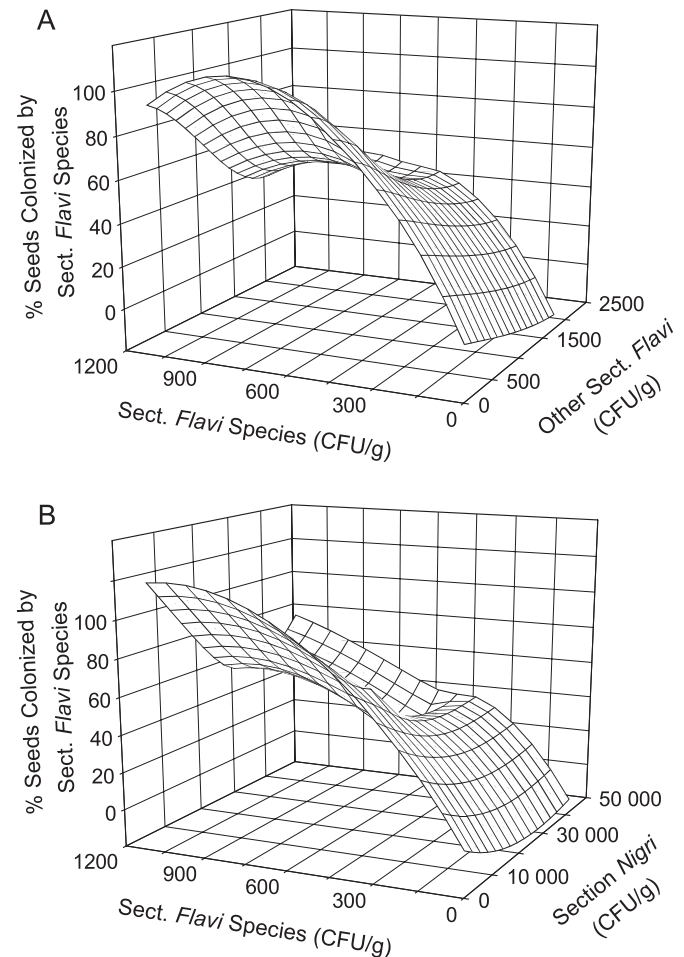


**Fig. 2.** Best-fit regression curves (exponential rise to maximum) for section *Nigri* and *Aspergillus terreus* (section *Terrei*) showing the effect of fungal density in 20 soils on the incidence of peanut seed colonization. Soil densities are based on the wet mass of the final soil preparation used for inoculating seeds; percentages of seeds colonized are based on 40 seeds per soil. Highest values for section *Nigri* (41 522 CFU/g, resulting in 100% of seeds colonized) and *A. terreus* (38 410 CFU/g, resulting in 100% of seeds colonized) are not shown. *Aspergillus* section *Nigri*:  $y = 93.87(1 - e^{-0.0038x})$ ,  $r^2 = 0.75$  ( $P < 0.0001$ ); *A. terreus*:  $y = 90.75(1 - e^{-0.0023x})$ ,  $r^2 = 0.93$  ( $P < 0.0001$ ).



fields resulted in a mean incidence of seed colonization by section *Flavi* of  $17.9\% \pm 23.26\%$  ( $\pm$ SD;  $n = 6$ ), whereas soils from cultivated fields with higher densities of section *Flavi* resulted in a mean incidence of  $85.0\% \pm 14.33\%$  ( $n = 14$ ). The relationship between soil density and the incidence of seed colonization in dominant *Aspergillus* species outside of section *Flavi*, specifically section *Nigri* and *A. terreus*, was also characterized by highly significant functions of exponential rise to maximum (Fig. 2). Section *Nigri* was co-dominant with section *Flavi* species on peanut seeds at 14 days following wound inoculation. *Aspergillus terreus*, though often present on seeds at high incidences, was gener-

**Fig. 3.** Quadratic regressions showing the combined effects of soil densities of section *Flavi* species and soil densities of competing *Aspergillus* species on the colonization of wounded peanut seeds by section *Flavi* species. Analyses were based on soil densities and seed incidences of individual species within section *Flavi* (6 species  $\times$  20 soils = 120).  $x_1$  = soil densities of six section *Flavi* species;  $x_2$  = soil densities of competing *Aspergillus* species;  $y$  = percentages of seeds colonized by six section *Flavi* species. (A) Competition with other section *Flavi* species (combined section *Flavi* species minus each individual species).  $y = 3.967 + 0.320x_1 - 0.000197x_1^2 - 0.00940x_2 + (4.224 \times 10^{-6})x_2^2 - 0.000113x_1x_2$ ,  $r^2 = 0.82$  ( $P < 0.0001$ ). (B) Competition with section *Nigri*.  $y = 3.992 + 0.236x_1 - 0.000112x_1^2 - 0.00142x_2 + (3.256 \times 10^{-8})x_2^2 - (2.272 \times 10^{-6})x_1x_2$ ,  $r^2 = 0.75$  ( $P < 0.0001$ ).



ally confined to the wound site early during the colonization of seeds (4 days) and was subsequently overrun by sections *Flavi* and *Nigri* species.

Competition among *Aspergillus* species in the colonization of peanut seeds was also examined statistically, since individual section *Flavi* species co-occurred in soil with other potentially competing members of section *Flavi* and with section *Nigri*. The combined effects of two independent variables (soil density of section *Flavi* species and soil density of competing *Aspergillus* species) on the incidence of seed colonization by section *Flavi* species were highly significant when competitors were other section *Flavi* species (section

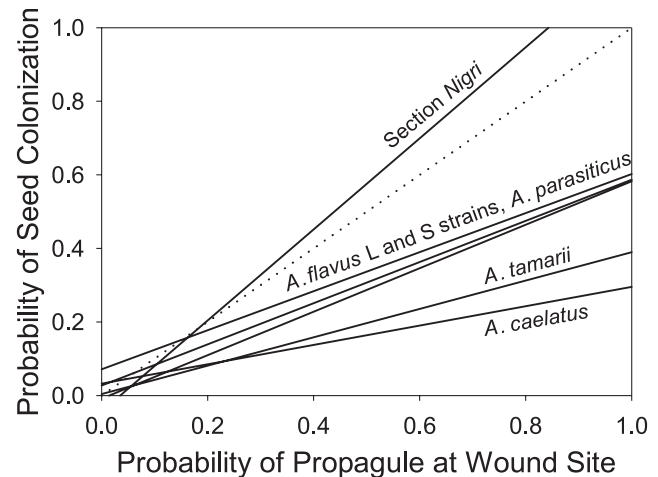
as a whole minus individual species) ( $F = 103.03$ ; 114 df;  $P < 0.0001$ ) and section *Nigri* ( $F = 70.06$ ; 114 df;  $P < 0.0001$ ) (Fig. 3). A significant interaction ( $F = 55.38$ ; 114 df;  $P < 0.0001$ ) was detected between the soil densities of section *Flavi* species and those of competing section *Flavi* species. Figure 3a shows that percent seed colonization by section *Flavi* species decreased with increasing soil densities of competing section *Flavi*; this pattern was most apparent at the higher soil densities of section *Flavi* species. The interaction between soil densities of section *Flavi* species and section *Nigri* was also significant ( $F = 16.13$ ; 114 df;  $P < 0.0001$ ). The effect of section *Nigri* on lowering seed colonization by section *Flavi* species (Fig. 3b) was less pronounced than that of competing section *Flavi* despite a much higher range of soil densities for section *Nigri*. For example, soil from Field 20 with the highest section *Nigri* density (41 522 CFU/g) resulted in 100% seed colonization by section *Nigri* (Fig. 2), yet section *Flavi* present at 1733 CFU/g in that same soil (Table 1) still colonized 92% of the peanut seeds.

### Estimations

Regression equations (Figs. 1 and 2; text for *A. alliaceus*) were used to estimate critical values for the relationship between soil propagule density and peanut seed colonization. Maximum incidences of seed colonization at saturation varied according to species. *Aspergillus flavus* S strain, section *Nigri*, and *A. terreus* were capable of colonizing seeds at high percentages (>90%), whereas *A. flavus* L strain, *A. parasiticus*, *A. caelatus*, and *A. tamarii* colonized seeds at relatively low maximal percentages (21%–66%) despite high densities in some soils (Figs. 1 and 2). Species also differed according to estimated fungal densities in soil required for colonization of 20% of peanut seeds (CFU/g): *A. parasiticus* (26), *A. caelatus* (29), *A. flavus* L strain (43), section *Nigri* (63), *A. flavus* S strain (88), *A. terreus* (110), *A. tamarii* (151), and *A. alliaceus* (219). Colonization of 20% of the seeds by each *Aspergillus* species occurred with an average of two or fewer propagules at the wound site, based on an estimated 6.8 mg of soil paste applied to the wound.

Soils estimated to result in <1 propagule of *Aspergillus* species at the wound site were treated as probabilities (e.g., 0.37 propagule at the wound implies that out of 100 seed inoculations, 37 will contain a propagule) and were plotted against actual probabilities of seed colonization (e.g., 43% of seeds colonized by a species indicates a probability of 0.43) (Fig. 4). The following linear regression equations ( $x$  = propagule probability in soil;  $y$  = actual seed colonization probability) and statistics were obtained: *A. flavus* L strain ( $y = 0.56x + 0.027$ ,  $n = 14$ ,  $r^2 = 0.54$ ,  $P < 0.01$ ), *A. flavus* S strain ( $y = 0.59x - 0.008$ ,  $n = 16$ ,  $r^2 = 0.84$ ,  $P < 0.0001$ ), *A. parasiticus* ( $y = 0.53x + 0.071$ ,  $n = 14$ ,  $r^2 = 0.53$ ,  $P < 0.01$ ), *A. caelatus* ( $y = 0.26x + 0.032$ ,  $n = 19$ ,  $r^2 = 0.41$ ,  $P < 0.01$ ), *A. tamarii* ( $y = 0.39x + 0.004$ ,  $n = 20$ ,  $r^2 = 0.81$ ,  $P < 0.0001$ ), and section *Nigri* ( $y = 1.24x - 0.043$ ,  $n = 6$ ,  $r^2 = 0.99$ ,  $P < 0.0001$ ). The  $y$  intercept in all equations was close to zero. The slopes were 0.53–0.59 for *A. flavus* L and S strains and *A. parasiticus*; 0.26 and 0.39 for *A. caelatus* and *A. tamarii*, respectively; and 1.24 for section *Nigri*. Linear regressions were not determined for *A. alliaceus* because few soils

**Fig. 4.** Linear regressions showing the relationship between the estimated probability of a propagule in soil at the wound site and the actual probability of seed colonization (see Results). *Aspergillus flavus* L and S strains and *A. parasiticus* have similar slopes and are labeled together. Broken line shows the equation  $y=x$  for reference.



contained the species nor were they determined for *A. terreus* because few soils resulted in <1 propagule at the wound site.

### Colonization by other fungal species

Several other *Aspergillus* species colonized peanut seeds but in a less consistent manner than section *Flavi* species, section *Nigri*, and *A. terreus* (Table 2). *Emericella rugulosa* (anamorph = *A. rugulovalvus*) occurred on seeds when inoculated with cultivated soils containing high densities of this species; Field 12 with the highest density of *Emericella rugulosa* (39 819 CFU/g) resulted in 97.5% seed colonization. *Emericella rugulosa* typically was overrun by sections *Flavi* and *Nigri* species and was often detectable only during the early stages of colonization (4 days). *Eurotium chevalieri* (anamorph = *A. chevalieri*) also colonized seeds only when inoculated with cultivated soils. Though the remaining *Aspergillus* species (Table 2) were often present in soils from all sources, the highest incidences of seed colonization by these species occurred with soils from forested locations and fallow fields in which the densities of sections *Flavi* and *Nigri* species were low. For example, locations with soils resulting in the highest percentages of seed colonization by *A. fumigatus* (25.0%, 42.0%, and 52.5%), *A. flavipes* (42.5%), and *A. melleus* (77.7% and 95.0%) were either forested or fallow. *Aspergillus japonicus*, *A. ustus*, *A. clavatus*, *A. kanagawaensis*, *A. carneus*, *Emericella nidulans*, and *Eurotium amstelodami* were characterized by low seed infectivity.

*Eupenicillium ochrosalmoneum* was the most prevalent fungal species on peanut seeds outside of the genus *Aspergillus* (Table 2). The species was detected only in soils from cultivated fields ( $n = 12$ ) and had a mean density of  $886 \pm 744.0$  CFU/g; the mean incidence of seed colonization was  $40\% \pm 32.2\%$  ( $n = 13$ ). *Eupenicillium ochrosalmoneum* was present on 159 seeds, and in all instances, the species was observed sporulating on the heads of section *Flavi* species in addition to sporulating directly on the seed. Other fungi that occurred

**Table 2.** Other fungal species colonizing peanut seeds.

Species	Soil		Peanut seeds	
	No. of fields populated by species	Population (CFU/g) <sup>a</sup>	No. of soils resulting in colonization	% seeds colonized
<b><i>Aspergillus</i> and its teleomorphs</b>				
<i>Aspergillus fumigatus</i>	20	1 – 15 796	7	2.5–52.5
<i>Aspergillus japonicus</i>	12	1 – 2 571	1	5.0
<i>Aspergillus ustus</i>	7	9 – 368	0	0
<i>Aspergillus flavipes</i>	6	1 – 395	3	2.5–42.5
<i>Aspergillus clavatus</i>	5	2 – 347	1	5.0
<i>Aspergillus carneus</i>	4	9 – 251	2	2.5–5.0
<i>Aspergillus kanagawaensis</i>	1	349	0	0
<i>Aspergillus melleus</i>	ND	ND	5	5.0–95.0
<i>Emericella rugulosa</i>	10	1 – 39 819	8	2.5–97.5
<i>Emericella nidulans</i>	1	183	0	0
<i>Eurotium amstelodami</i>	ND	ND	1	5.0
<i>Eurotium chevalieri</i>	ND	ND	5	3.6–62.5
<b><i>Penicillium</i> and its teleomorphs</b>				
<i>Penicillium citrinum</i>	—	—	2	22.5–30.0
<i>Penicillium</i> cf. <i>vinaceum</i>	—	—	2	5.0
<i>Penicillium pinophilum</i>	—	—	1	2.5
<i>Penicillium</i> cf. <i>rugulosum</i>	—	—	1	87.5
<i>Penicillium</i> spp.	—	—	13	2.5–82.5
<i>Eupenicillium ochrosalmoneum</i>	12	230 – 2 686	13	7.5–89.3
<b>Other genera</b>				
<i>Alternaria alternata</i>	—	—	2	2.5–22.5
<i>Paecilomyces fulva</i>	—	—	2	2.5–32.5
<i>Syncephalastrum racemosum</i>	—	—	4	2.5–7.5
<i>Cunninghamella elegans</i>	—	—	2	2.5
<i>Absidia cylindrospora</i>	—	—	1	2.5

**Note:** ND, not detected in soil. —, not quantified in soil.

<sup>a</sup>Densities are based on wet mass of final soil preparation used for inoculating seeds.

on >20% of seeds included *Penicillium citrinum*, *Penicillium* cf. *rugulosum*, *Alternaria alternata*, and *Paecilomyces fulva*. The highest incidences of these fungi on seeds were all associated with forested locations and fallow fields whose soils contained low densities of sections *Flavi* and *Nigri* species.

## Discussion

Soils used for inoculating seeds varied considerably in population densities of *Aspergillus* species. The high densities of section *Flavi* species in soils from cultivated fields relative to those from fallow fields and forested locations can be attributed to the influx of inoculum from infected crops over years of cultivation (Horn 2005a). Wounded peanut seeds in the present study were maintained under conditions (seed  $A_w = 0.92$  at 37 °C) conducive for colonization from soil by section *Flavi* species (Horn 2005b).

The relationships between soil population density and the incidence of seed colonization by *Aspergillus* species were best characterized by exponential functions in which an increase in soil density resulted in an increase in seed colonization. Exponential curves often rose to maximum

percentages of seed colonization by section *Flavi* species that were well below 100% despite high species densities in some soils. Competition of individual section *Flavi* species with other dominant colonists of seeds, primarily other species belonging to section *Flavi* as well as section *Nigri*, may explain in part the low maximum incidences of some section *Flavi* species on peanut seeds. Seed colonization by individual species within section *Flavi* decreased with increasing soil densities of competing species from the same section. Competitive displacement of section *Flavi* species by the same and other species in the section has been demonstrated in current practices of biological control in which nontoxigenic strains of *A. flavus* and *A. parasiticus* applied to soil in high conidial numbers reduce aflatoxins by excluding native aflatoxigenic strains from peanut seeds (Dorner 2004; Dorner et al. 1998; Dorner and Horn 2004). *Aspergillus niger*, a species within section *Nigri*, commonly co-occurs with section *Flavi* species on agricultural commodities, including peanuts (Abdalla 1974; Joffe 1969). In the present study, section *Nigri* also co-occurred with section *Flavi* species on peanut seeds but was less inhibitory than competing section *Flavi* species toward the colonization of seeds by section *Flavi*

species. *Aspergillus niger* has been shown to inhibit aflatoxin production under laboratory conditions (Wicklow et al. 1980; Horn and Wicklow 1983) and in cultivated corn and peanuts (Hill et al. 1983; Wicklow et al. 1988a). However, the application of large numbers of *A. niger* conidia to drought-stressed peanuts for biological control fails to reduce aflatoxins (Dorner 2004). This observation is supported by the current study in which high soil densities of section *Nigri* resulted in low inhibition of seed colonization by section *Flavi* species.

*Aspergillus flavus* and *A. parasiticus* have the capacity to invade both dead and living plant tissue (Diener et al. 1987; Payne 1998). Horn (2005b) concluded that invasion of living seed tissue has features more characteristic of saprotrophy than parasitism, since the inoculation of wounded viable and nonviable (autoclaved) peanuts seeds with soil results in similar colonization patterns by section *Flavi* species. Garrett (1970) defined competitive saprotrophic (saprophytic) ability as the "summation of physiological characteristics that make for success in competitive colonization of dead organic substrates." Competitive saprotrophic ability is complex and is influenced by environmental factors (e.g., substrate composition, temperature, moisture) and factors intrinsic to the fungus (e.g., propagule type, growth and spore germination rates, enzyme production). In this study, the soil density of species estimated to colonize 20% of wounded peanuts may be a measure of the competitive saprotrophic ability of species within section *Flavi*, with those species requiring lower densities for colonization being the most aggressive. *Aspergillus flavus* L strain and *A. parasiticus* with low soil density requirements for colonization are the most frequently isolated section *Flavi* species from peanut seeds grown under field conditions (Diener et al. 1987; Hill et al. 1983). *Aspergillus caelatus*, also with a low density requirement, is a recently described species and its incidence on peanuts is not well documented (Horn 1997; Horn and Greene 1995). *Aspergillus flavus* S strain with a slightly higher density requirement is infrequent in soils from peanut-growing regions in the United States (Horn and Dorner 1998). The remaining members of section *Flavi*, i.e., *A. tamarii* and *A. alliaceus*, have high soil density requirements and are infrequently observed in peanuts (Moubasher et al. 1979; Pildain et al. 2004).

Peanut seeds are often preferentially colonized by section *Flavi* even though soil densities of the section relative to total numbers of filamentous fungi are usually <1% (Horn 2005b). Low propagule numbers of *Aspergillus* species in soil, averaging two or fewer propagules at the wound site, were required for colonization of 20% of peanut seeds and, therefore, support the conclusion of Griffin and Garren (1974) that as few as 2.0 propagules of *A. flavus* in the 0.5 mm soil layer of the geocarposphere surrounding the peanut pod are required for infection. Conidia of *A. flavus* are quiescent in the geocarposphere of peanut pods because of soil fungistasis but germinate readily in response to pod injury and the subsequent release of sugars and N-amino compounds (Griffin 1972; Hale and Griffin 1976).

The efficiency of low propagule numbers in colonizing wounded peanut seeds also was examined in the present research. The probability of seed colonization (based on actual incidences of seed colonization) when regressed on the probability of propagules being present in soil at the

wound site (based on soils showing <1 propagule per wound) should give a slope of 1 if there is an exact correspondence between the two. Section *Nigri* with a slope of 1.24 approximated this degree of correspondence. Slopes of 0.53–0.59 for *A. flavus* L and S strains and *A. parasiticus* suggest that approximately half of the propagules in soil resulted in colonization of the peanut seeds. Slopes of 0.26 and 0.39 for *A. caelatus* and *A. tamarii*, respectively, suggest even less efficiency in colonization from soil. The necessity of subculturing section *Flavi* from seeds for identification to species (section *Nigri* was visually detected directly on seeds) may have resulted in an underestimate of seed colonization due to an inability to detect low levels of sporulation among co-occurring section *Flavi* species. The importance of this potential source of error in analyzing the efficiency of soil propagules for seed colonization is not known.

Horn (2005b) inoculated wounded peanut seeds with two soils and incubated the seeds at different combinations of temperature and water activity. Because both soils were from cultivated fields, they contained sizable populations of sections *Flavi* and *Nigri* species. It was concluded that other fungal species in soil colonize seeds primarily at temperatures and seed water activities suboptimal for more aggressive sections *Flavi* and *Nigri* species. In the current study, other fungal species were capable of invading peanut seeds under optimal conditions for sections *Flavi* and *Nigri* but only when soil densities for these sections were low. The dominant species outside of the genus *Aspergillus*, *Eupenicillium ochrosalmoneum*, produces the neurotoxin citreoviridin (Wicklow et al. 1988b) and has previously been reported to sporulate on the heads of section *Flavi* species (Horn 2005b). The restriction of *Eupenicillium ochrosalmoneum* to soil primarily from cultivated fields is likely due to infection of corn and peanuts by this fungus (Horn 2005b; Wicklow et al. 1984) and the release of ascospores from ascostromata dispersed onto soil (Horn and Wicklow 1986).

Optimal temperatures for *A. flavus* in the colonization of wounded peanut seeds inoculated with soil are 30–37 °C (Horn 2005b), temperatures that are typical of peanut field soils under conditions of late-season drought in tropical and subtropical regions (Hill et al. 1983; Waliyar et al. 2003). In contrast, the optimal temperature for *A. parasiticus* and *A. caelatus* is approximately 22 °C, although both species can also colonize seeds at 37 °C (Horn 2005b). The present experimental conditions (seed  $A_w = 0.92$  at 37 °C) are, therefore, most conducive to invasion by *A. flavus*, and the lower temperature optimum for *A. parasiticus* and *A. caelatus* may reduce their competitive saprotrophic ability at 37 °C. This may account for the relatively low maximum percent seed colonization for these two species. Examination of a full range of environmental conditions is required for an accurate analysis of the relationship between soil density of section *Flavi* species and colonization of peanut seeds.

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